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Eur päisches Patentamt  
European Patent Office  
Office européen des brevets

11 Publication number:

**0 142 193**  
**A1**

12

## EUROPEAN PATENT APPLICATION

21 Application number: 84201502.6

22 Date of filing: 17.10.84

51 Int. Cl.<sup>4</sup>: **A 61 K 39/385**  
**C 07 G 3/00, C 07 K 17/10**  
**G 01 N 33/548**

30 Priority: 22.10.83 NL 8303646

43 Date of publication of application:  
22.05.85 Bulletin 85/21

84 Designated Contracting States:  
BE CH DE FR GB IT LI LU NL SE

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54 Preparation of immunogens consisting of antigens and/or antigenic determinants bound to glycoside-containing carriers.

57 The invention concerns a method for the preparation of immunogens comprising reacting at least one type of antigens or antigenic determinants which are for example of polypeptide, oligosaccharide or oligonucleotide nature, with carrier complexes consisting of glycosides having a hydrophilic and a hydrophobic part and hydrophobic compounds provided with one or more groups which show reactivity towards said antigens or antigenic determinants. These immunogens are useful as vaccines, and as immunochemical reagents. Prior to use the immunogens may be stabilized (e.g. by cross-linking of the antigens or antigenic determinants) and/or be protected against digestive degradation (e.g. by micro- or macro-encapsulation).

**EP 0 142 193 A1**

PREPARATION OF IMMUNOGENS CONSISTING OF ANTIGENS  
AND/OR ANTIGENIC DETERMINANTS BOUND TO GLYCOSIDE-  
CONTAINING CARRIERS.

The invention relates to the preparation of immunogens consisting of antigens and/or antigenic determinants bound to a carrier and vaccines which contain these immunogens.

5        Immunization is employed extensively as a way to protect man and animals against infections by pathogenic organisms such as viruses, bacteria, parasites and the like. Active immunization, which provides long-lasting protection, is preferred.

10        Active immunization is effected by introducing antigens of the pathogenic organism into the individual to be protected. Mostly this is done by inoculation with inactivated or attenuated strains of the pathogen.

Inoculation with inactivated strains has the disadvantage that the immunizing power is often rather low due to the partial denaturation of the antigens which occurs during inactivation.

- 5 Inoculation with attenuated strains of the pathogens entails the risk of a certain pathogenicity of these strains.

- Hence, a safer and more efficient method of active immunization is desired. One possibility is, for example, inoculation with preparations which exclusively contain antigens instead of the complete pathogenic organisms. In this case, the achievement of very high purity, and in particular the complete removal of, for example, viral genetic material, is often difficult to attain. As more information continues to become available on the primary and spatial structure of the antigens, ways have been sought of using antigen fragments which are as small as possible, the so-called antigenic determinants, as immunogens for active immunization. An antigenic determinant is a part of the antigen which can act as a recognition site for a particular type of immunoglobulin directed against the antigen. When split off from the complete antigen, such small fragments are often easier to purify than the antigen itself, and are even small enough to permit their synthetic preparation.

- 30 However, a major drawback remains that inoculation with freely dissolved antigens and antigenic determinants often results in much too low a level of immunisation.

Significantly better immunization has been achieved by inoculation with an immunogen wherein antigens or antigenic determinants are bound to a carrier. Specifically, known carriers for this purpose are immunogenic macromolecules such as tetanus toxoid, key-hole limpet haemocyanin (KLH), multi-poly(DL-alanyl)-poly(L-lysine) and copolymers of D-aminoacids. The antigens or antigenic determinants, thus rendered more strongly immunogenic, are however still somewhat less immunogenic than, for example, the antigens in their natural form, such as intact micro-organisms. Moreover, the macromolecular carriers employed therein are less suitable for human administration and, for example, KLH is commercially hardly attractive because the material is so expensive.

There have now been found novel immunogens which appear to possess substantially better immunizing power than the immunogens wherein the carriers hitherto known are present. The carriers employed in the immunogens according to the invention are moreover non-pyrogenic and commercially more attractive than the known macromolecular carriers.

The preparation of the immunogens according to the invention is characterized in that antigens or antigenic determinants or derivatives thereof are bonded to reactive groups of hydrophobic compounds which are complexed with at least one type of glycosides having a hydrophilic and a hydrophobic part, whereafter the resulting immunogenic particles are optionally stabilized and/or protected against digestive degradation.

Any glycoside having a hydrophobic part and a hydrophilic part can be used.

Preferably, saponins are used, such as those described by Tschesche and Wulf, "Chemie und Biologie der Saponine" in Fortschritte der Chemie organischer Naturstoffe, edited by W. Herz, H. Grisebach and G.W. Kirby, Vol. 30 (1973) and in particular the highly polar saponins, and especially the polar triterpene-saponins such as the polar acidic bis-desmosides, for example saponin-extract from Quillaja bark, araloside A, Chikusetsu-saponin IV, Calendula-glycoside C, Chikusetsu-saponin V, Achyranthes-saponin B, Calendula glycoside A, araloside B, araloside G, Putranjia-saponin III, Bersama saponoside, Putranjia-saponin IV, trichoside A, trichoside B, saponoside A, trichoside C, gypsoside, nutanoside, dianthoside C, saponoside D, aescine from Aesculus hippocastanum or saponoside from Gypsophilla struthium, and in particular saponin extract from Quillaja saponaria Molina, preferably the DQ-extract prepared by the method of K. Dalsgaard ("Saponin Adjuvants", Bull.Off.int. Epiz 77 (7-8), 1289-1295 (1972)) and Quil A prepared by the method of K. Dalsgaard ("Saponin Adjuvants III", Archiv für die gesamte Virusforschung 44, 243-254 (1974)).

Mixtures of glycosides may also be used.

According to the present invention antigens or antigenic determinants are used in the preparation of immunogens.

As antigens can be used macromolecular compounds like proteins or polypeptides, polysaccharides or oligosaccharides, glycoproteins, lipoproteins and polynucleotides. These antigens

may be naturally occurring as such, or may be obtained from organisms, in particular from viruses, bacteria, eukaryotic microorganisms, parasites or cells or cell lines belonging to or derived from multicellular organisms. Optionally these antigens may be cleaved enzymatically or chemically prior to use, e.g. in order to remove possibly interfering groups, or to raise the antigenicity. In particular the antigens may also be obtained from recombinant organisms; more particularly recombinant DNA encoded polypeptides are very suitable antigens.

Antigenic determinants suitable for preparing immunogens according to the invented process are for example small polypeptides, small oligosaccharides, oligonucleotides, glycoprotein fragments and haptens.

With small polypeptides are meant here polypeptides comprising at least 4 up to about 40 aminoacids. In general an antigenic determinant comprises not more than 4-8 amino acids. Sometimes, however, a somewhat larger number of amino acids is needed in order to ensure the specific spatial structure of the antigenic determinant. The polypeptides may for example be prepared synthetically on the basis of a known amino acid sequence, or may be obtained by proteolytic or chemical cleavage of larger polypeptides or proteins such as membrane proteins of microorganisms or proteins of plant or animal origin or may be a polypeptide hormone or a fragment or analogue thereof.

With small oligosaccharides in general are meant linear or branched chains of sugar units comprising at least 4 and up to about 20 sugar units, and more favorably between 6 and 10 sugar units. These oligosaccharides can be prepared synthetically or by chemical or enzymic degradation of naturally occurring polysaccharides or glycoproteins.

With oligonucleotides are meant compounds consisting of at least 1 up to about 18 deoxyribonucleotides or ribonucleotides which are obtained synthetically, or by enzymatic or chemical cleavage of polynucleotides such as RNA and DNA. Optionally these oligonucleotides may be double-stranded.

Glycoprotein fragments are prepared synthetically or by chemical or enzymatic cleavage of glycoproteins.

With haptens are meant molecules different from the polypeptides, oligosaccharides, oligonucleotides and glycoprotein fragments mentioned before and which are not suited for the induction of antibody formation unless bonded to a carrier. Examples of these are steroids and prostaglandins.

The antigens or antigenic determinants are to possess at least one reactive group which is suited to directly or indirectly form a bond between said antigen or antigenic determinant and the hydrophobic compounds complexed with the glycosides.

As hydrophobic compound can be used any chemical group with hydrophobic character. Examples of these are hydrophobic polypeptides, or aliphatic compounds or phospholipid derivatives, or cationic detergents.

Hydrophobic polypeptides are suitable hydrophobic compounds in the immunogen according to the invention. These polypeptides can preferably comprise 4-50 amino acids and can in particularly consist of hydrophobic amino acid residues, such as valyl, phenylalanyl, tryptophyl, methionyl, leucyl and/or isoleucyl residues. Inter alia in the interests of appropriate solubility, for example in the course of the synthesis, it may be necessary to include a number of additional hydrophilic amino acids in the hydrophobic compound.

Suitable hydrophobic compounds further are aliphatic straight or branched chain hydrocarbon derivatives, such as alkylamines having about 5-18 carbon atoms, or alkylmercaptans having about 4-15 carbon atoms.

Suitable also are cationic detergents, such as DTAB, DTAC or OTAC, containing additional reactive groups like sulfhydryl, hydroxyl or carboxyl groups.

Advantageously also phospholipids, such as phosphatidyl derivatives containing straight or branched hydrocarbon chains may be used as hydrophobic compounds.

Whichever of the above or other hydrophobic compounds are selected, a general prerequisite is the presence of at least one reactive group such as for example an amino, carboxyl, sulfhydryl, hydroxyl, aldehyde or ketone group. These groups either may form part of the hydrophobic compounds or may be linked thereto by means of a linker following formation of the hydrophobic complex. The nature of the reactive group actually selected primarily depends on the nature of the reactive



groups on the antigen or antigenic determinant to be reacted therewith.

5       The complexes of the hydrophobic compound  
and the glycosides with hydrophilic and hydro-  
phobic parts (carrier complexes) can be prepared  
in numerous possible ways. Stated in most general  
terms, a solution of the hydrophobic compounds  
to which, if desired, a solubilizing agent is  
10       added, is brought into contact with a glycoside  
solution which contains at least one glycoside  
which is composed of one hydrophobic and one  
hydrophilic part, in an amount at least equal to  
the critical micelle concentration, and at the  
15       same time or subsequently any solubilizing agent  
present is removed.

Such a preparation can be carried out by  
dialysing or subjecting to ultrafiltration a  
solution of the hydrophobic compound, together  
20       with a solubilizing agent in the presence of the  
said glycoside solution.

Specifically for the large-scale preparation  
of the carrier complex according to the invention,  
ultrafiltration is a very suitable method.

25       Advantageously the carrier complex may be  
prepared by mixing both hydrophobic compound and  
glycosides with hydrophilic and hydrophobic parts  
in a solvent and applying sonication to this  
mixture. The carrier complexes subsequently may  
30       be separated and/or purified.

However, it is also possible to prepare the  
carrier complexes by introducing a solution of the  
hydrophobic compound, in the presence of a  
solubilizing agent, onto a density gradient of,  
35       for example, sugar solutions or salt solutions,

to which such an amount of glycoside containing a hydrophobic part and a hydrophilic part is added that the concentration is at least equal to the critical micelle concentration, and  
5 thereafter introducing the whole into a centrifugal force field, whereby the carrier complex is separated out as a band.

The hydrophobic compounds within the carrier complexes thus prepared may or may not  
10 be provided with reactive groups suitable for binding of the antigens or antigenic determinants. If reactive groups are present it has to be made sure, by a proper choice of the groups and/or by a proper way of preparing the complexes, that  
15 these reactive groups are actually accessible to subsequent coupling of antigens or antigenic determinants.

In particular this can be done using hydrophobic compounds to which the reactive groups are  
20 attached via more or less hydrophilic linker groups.

Alternatively the reactive groups may be bonded to the hydrophobic compounds subsequent to the formation of the carrier complexes.  
25 Advantageously this can be done by using a bifunctional linker, which on one side attaches to the hydrophobic compound within the complex and on the other side is suited for attachment of the antigen or antigenic determinant.

30 Suitable bifunctional reagents for cross-linking are described by T.H. Ji (Methods in Enzymology 91, 580-609 (1983)).

As a bifunctional linker advantageously use may be made of a hetero-bifunctional reagent. More in particular are suited bifunctional reagents having one reactive site which reacts  
5 with an amino group (such as a succinimidyl group or an imidate group) and another reactive site which reacts with a different group such as a sulfhydryl group (e.g. a maleimido group) or with various groups (e.g. an azidophenyl  
10 group, which reacts upon UV radiation) or a dithio pyridyl group.

The invention also includes the preparation of immunogens which comprise various types of antigens and/or antigenic determinants. These  
15 can be different antigens and/or antigenic determinants which are characteristic of one and the same antigenic and/or pathogenic entity, if desired for example characteristic for different serotypes of the same pathogen, or  
20 different antigens and/or antigenic determinants which are characteristic of different antigenic and/or pathogenic entities.

The immunogens according to the invention can be stabilized, if desired. This can be done,  
25 for example, by linking to themselves, or to one another, the antigenic determinants and/or the hydrophobic residual groups bonded thereto ("cross-linking").

The cross-linking can be established by any  
30 reagent possessing reactive groups which are able to bond to at least two groups on the antigen or antigenic determinant and/or on the hydrophobic residual group. For the cross-linking of polypeptides generally use is made of glutaraldehyde  
35 or formaldehyde. Suitable cross-linking agents

are also homobifunctional reagents (such as bisimides, bisuccinimidyl esters) or hetero bifunctional reagents (such as compounds which contain an azidophenyl group and an amide  
5 or a succinimidyl or an activated fluorobenzene or a haloketon group).

The immunogen according to the invention is in particular suitable for vaccination. A vaccine containing the said immunogen can  
10 inter alia be used for immunization against a particular pathogen, for so-called priming (wherein the body is not immediately stimulated to form specific free antibodies but is effectively preconditioned so that, following infection or  
15 vaccination, for example with inactivated or attenuated pathogens, a strong immunisation reaction is brought about; see, inter alia, Emini et al. (1983) Nature 304, 699-703), or for immunological sterilization or castration (whereby  
20 antibodies against substances vital to reproductive processes, such as certain hormones, are produced).

The invention therefore also includes vaccines for animal or human use which contain immunogens according to the invention. Such  
25 vaccines can be suitable for immunization or for priming against a particular pathogen, with at least one type of antigen or antigenic determinant characteristic of the particular pathogen being present in the immunogen. A plurality of types of  
30 antigens and/or antigenic determinants of one and the same pathogen can also be present in the vaccine. At the same time it is possible for each immunogen to comprise a plurality of these antigens and/or antigenic determinants, or for each of the  
35 types of antigens and/or antigenic determinants to

be present in separate immunogens. It is also possible, according to the invention, to compose vaccines for a plurality of pathogens, and in that case, once again, the respective antigens and/or antigenic determinants can be present in separate immunogens or conjointly in one immunogen.

The immunogen according to the invention can accordingly also be used in diagnostic tests, for example as a solid phase in a so-called sandwich test, as a particulate phase in an agglutination test, or as a marker phase in an immunochemical test; in the case of the last-mentioned use, the marker is, or can be, included in the immunogen.

To use the immunogen according to the invention for vaccination purposes, the immunogen must be brought into a suitable form for administration. For intramuscular or subcutaneous administration, the immunogens can, for example, be mixed with physiological salt solution. For intranasal or intraocular administration, for example in the form of a spray, an aqueous solution will also preferably be used. For local (for example oral) administration it will in many cases be necessary to use a form which temporarily protects the immunogens (for example against saccharolytic enzymes in the oral cavity or against proteolytic enzymes in the stomach). Such protection may for example be effected by encapsulating the immunogens or by bringing them into a slow-release form or by using one or more aminoacids in the D-form on enzyme-sensitive sites of the polypeptide antigens or antigenic determinants and/or hydrophobic compounds.

By encapsulation is meant any method by which the pharmaceutical is (temporarily) protected against attack by certain desintegrating influences. Encapsulation may be established by covering any  
5 individual complex according to the invention with a protecting agent (microcapsules), or by encasing a multitude of complexes into a single protecting shield (macrocapsules).

The encapsulating material may be semi-  
10 permeable or may become (semi-)permeable if brought within the animal or human body or upon attaining certain sites within the animal or human body. In this way both encapsulation and sustained release may be established by a single  
15 pharmaceutical preparation. For the encapsulation, therefore, generally a biodegradable material is used.

For the micro-encapsulation suitably the optionally cross-linked complexes according to  
20 the invention are dispersed into a solution of a biodegradable polymer, to which, optionally, surfactants are added. Consequently the polymer is to be build a capsule at the interface. The microcapsules are separated and might be suspended  
25 into a physiologically acceptable medium. Alternatively the microcapsules may be lyophilized.

Macro-encapsulation can be established by methods known in the art. Suitably the optionally cross-linked, complexes according to the invention  
30 are converted into an appropriate formulation compatible with oral (soft or hard) capsule material, such as gelatine, starch, etc. To this end the complexes to which are added for example mannitol, sucrose, glycerol, etc., may be dried  
35 or freeze-dried, whereafter the material can be

granulated or suspended into for example a natural or synthetic gel and filled into capsules which have been enteric coated by, for example, formaldehyde, cellulose acetate phtalate, hydroxypropylmethyl cellulose phtalate or polyacrylic compounds. Alternatively enteric coated capsules are covered internally with a soft hydrophobic layer such as polysiloxane or synthetic or vegetable oils or waxes, prior to filling with the complexes according to the invention in aqueous suspended form.

The invention is further illustrated by reference to the examples below.

15

#### Example 1

##### IBV protein immunogen

##### A. Preparation of IBV antigen:

Avian infectious bronchitis virus (IBV) belonging to the group of Corona viruses is purified from the allantoic fluid of IBV-infected 11 day old embryonated eggs by sucrose gradient centrifugation.

The purified IBV is suspended in PBS at 5 mg protein per ml and treated with thermolysin (0.1 mg/ml) in the presence of 5 mM  $\text{CaCl}_2$  for 15 minutes at 22 °C. The enzymic reaction is stopped by adding 4 mM EDTA (final concentration). By this enzymic reaction the proteineous spikes at the surface of the virus are cut off.

The spikes are separated from the virion particles by centrifugation for 60 minutes at 100.000 xg. The supernatant containing the solubilized spike proteins are diafiltrated in an Amicon ultrafiltration cell equipped with a

PM 30 filter, and subsequently lyophilized.

B. Preparation of saponin Quil A carrier complex:

A concentrated solution of the thiocholine

N-n-dodecyl-N,N-dimethyl-N-(2-thioethyl)-

5 ammoniumchloride $[n-C_{12}H_{25}^+N(CH_3)_2-CH_2-CH_2-SH.Cl^-]$ ,

in aqueous ethanol (1:1), is added to a 1%

solution of the saponin Quil A in deoxygenated

0.1 M phosphate buffer, pH 6.0, 0.9% in NaCl,

5 mM in EDTA, to a final concentration of

10 0.4 mM in said thiocholine surfactant. Mixed

micelles are formed.

C. Activation of carrier complex:

The mixed micelles prepared according to B.

are reacted with 5 mM N-succinimidyl-4-(N-

15 maleimido-methyl)-cyclohexane-1-carboxylate

(SMCC) in 0.1 M phosphate buffer pH 5.0, 0.9%

in NaCl, 5 mM in EDTA for 30 minutes at room

temperature. Excess SMCC is removed by gel

filtration on Sephadex G 25 in 0.1 M phosphate

20 buffer, pH 5.0, 0.9% NaCl.

D. Preparation of immunogens:

The activated carried complex obtained under C.

is promptly added to a 10 mg/ml solution of the

IBV antigen obtained under A., in 0.1 M phosphate

25 buffer, pH 7.4, 0.9% NaCl. The resulting mixture

contains Quil A and protein in a weight ratio of

1 to 4. The pH of the solution is adjusted to 7.8

with 0.1 N NaOH, and the mixture is left at room

temperature for 2 hours. The immunogens are

30 isolated by centrifuging through a 10-40% by

weight of a sucrose gradient at 250,000 g for

10 hours at +4 °C. The complex is then dialysed

against 20 mM phosphate buffer pH 7.4, 150 mM

NaCl. Particulate immunogen complexes are

35 visible with the electron microscope.



Example 2Parvovirus immunogen I

## A. Preparation of porcine parvovirus antigen:

The 65 K MW major capsid protein of porcine  
5 parvovirus (PPV) is isolated according to the  
method described by Molitor, T.W., Joo, H.S. and  
Collett, M.S.: J.Virology 45, 842-854 (1983):  
PPV containing tissue culture fluid is brought  
to 25 mM  $\text{CaCl}_2$  by dropwise addition of concen-  
10 trated  $\text{CaCl}_2$  solution. The virus precipitate  
is spun for 20 minutes at 120,000 xg. The virus  
containing pellet is subjected to CsCl gradient  
centrifugation and the virus peak at a density  
of 1.3 g/ml is pooled, dialysed and treated with  
15 1% sodium dodecyl sulphate (SDS) for 3 minutes.  
The 65 K protein is isolated by preparative  
SDS-PAGE, dialysed and freeze-dried.

## B. Derivatization of PPV protein:

The protein obtained under A. is dissolved  
20 in 0.1 M phosphate buffer, pH 7.5, at 3 mg/ml  
and treated with 5 molar equivalents of SMCC at  
room temperature for 30 minutes. Excess SMCC is  
removed by gel filtration on Sephadex G 25 in  
0.1 M phosphate buffer, pH 6.0, 0.9% NaCl, 5 mM  
25 EDTA.

## C. Preparation of carrier complex:

The  $\text{N}^\alpha$ -3-(2-pyridyldithio)propionylated  
methyl ester derivative of the peptide Lys-Pro-Val-  
Gly-(Leu)<sub>5</sub>-Gly in trifluoro-ethanol solution  
30 (4%, w/w) is added to a 1% (w/w) solution of the  
saponin Quil A in 0.1 M acetate buffer, pH 4.5.  
The resulting mixture, being 2 mM in peptide, is  
then sonicated to obtain a clear solution.  
EDTA is added to 5 mM and the solution is flushed  
35 with nitrogen. Dithio erythritol is added to a

concentration of 20 mM. The reduction is allowed to take place for 15 minutes at room temperature, upon which the excess of DTE is removed by gel filtration over a Sephadex column equilibrated with a deoxygenated 0.1 M phosphate buffer, pH 6.0, 0.9% in NaCl, also containing 5 mM EDTA.

D. Preparation of immunogens.

The derivatized protein obtained under B. is combined with the thiolated carrier complex obtained under C. in a ratio of 1:4 (Quil A: protein). The solution is left for 5 hours at room temperature, whereon the immunogen complex is isolated by centrifuging in a 10-40% (w/w) sucrose gradient at 250,000 xg for 10 hours at +4 °C. The immunogen complexes are subsequently dialysed against 20 mM phosphate buffer pH 7.4, 150 mM NaCl.

Example 3

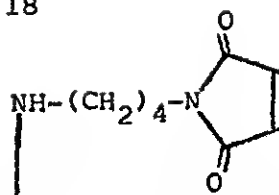
Parvovirus immunogen II

Similar to Example 2 a parvovirus immunogen is prepared starting from the PPV 65 K protein derived by molecular cloning of the respective virus gene in an expression vector in E.coli (Amgen, California, U.S.A.).

Example 4

β-endorphin-(6-17) immunogen

1.2 molar equivalents, with respect to the thiocholine compound of a maleimide derivative of β-endorphin-(6-17),



H-Thr-Ser-Glu-Lys-Ser-Glu-Thr-Pro-Leu-Val-Thr-Leu-OH,  
dissolved in a 0.1 M phosphate buffer, pH 6.0,  
5 0.9% in NaCl, 5 mM in EDTA, are added to the  
saponin Quil A carrier complex prepared according  
to Example 1 B.

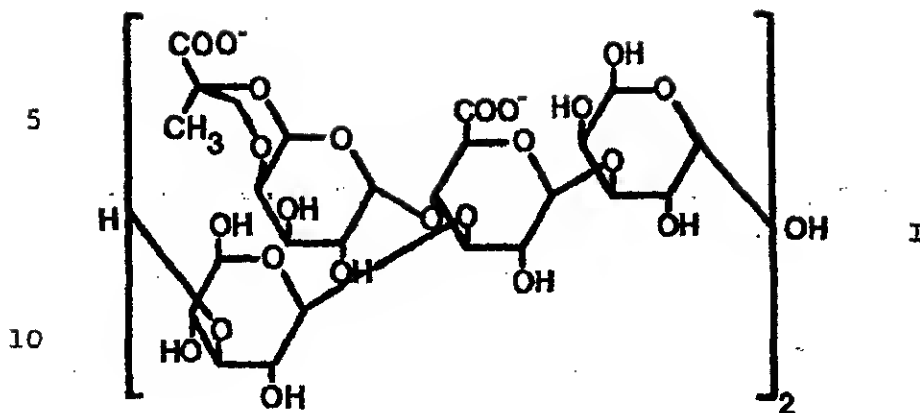
The mixture is kept at room temperature for  
1 hour and is subsequently dialysed against three  
10 changes of 20 mM phosphate buffer, pH 7.4, 150 mM  
in NaCl for 15 hours at 4 °C.

#### Example 5

##### Klebsiella serotype 11 immunogen

15 A. Klebsiella serotype 11 capsular octasaccharide.

An octasaccharide characteristic of the  
Klebsiella serotype 11 capsular polysaccharide  
(K 11 PS) is prepared according to Zigterman et al.  
(XIIth Internat. Carbohydrate Symp. at Utrecht,  
20 the Netherlands; Vonk Publishers, Zeist, 1984;  
Ed. Vliegenthart et al. p. 308). In summary 5 mg of  
K 11 PS to which  $5 \times 10^{10}$  pfu of the bacteriophage  
Ø 11 in 6 ml of PBS is added is incubated during  
24 hours at 37 °C. The octasaccharide (K 11 OS)  
25 subsequently is isolated and purified by gel  
filtration on Sephadex G 25 (Pharmacia). An octa-  
saccharide of the formula I is obtained.



B. Activation of K 11 OS

15        20 mg of the octasaccharide prepared under A. are reacted during 10 days with 25 mg of 2-(4-aminophenyl)-ethylamine while the resulting labile N-alkyl derivative is reduced with sodium cyanoborohydride in 2 ml of aqueous buffer, pH 8.5.

20    After removal of the excess of the reactants by gel permeation chromatography, the product is treated with excess thiophosgene and the resulting isothiocyanatophenyl derivative is freeze-dried.

C. Preparation of carrier complex.

25        25 mg of phosphatidyl ethanolamine are mixed with 175 mg of the saponin Quil A in a 1% solution in 0.15 M phosphate buffer of pH 7.4 and sonicated during 20 minutes at room temperature. The resulting complexes are extensively dialysed

30    against 0.1 M bicarbonate buffer, pH 9.

D. Preparation of immunogen.

      The activated oligosaccharide obtained under B. is dissolved in the carrier complex solution obtained under C. and reacted during 1 week. The product is

35    dialyzed against three subsequent changes of PBS and

freeze-dried.

Example 6

Cross-linked IBV immunogen

- 5        To the IBV immunogen prepared according to  
Example 1 suspended at about 5 mg/ml in 2 ml of  
0.1 M phosphate buffer, pH 7.5 is added dropwise,  
under continuous stirring 1 ml, 20 mM glutar-  
aldehyde. The reaction mixture is left for  
10    30 minutes at room temperature and then chilled  
in ice. The cross-linked complexes are freeze-  
dried after extensive dialysis against the above  
described buffer.

15

Example 7

Encapsulated canine parvovirus immunogen

- Immunogens containing canine parvovirus (CPV)  
64 k capsid protein are prepared according to the  
method described in Example 2.
- 20        0.15 g of the above immunogen in 8 g of a 21%  
albumin solution are mixed with 20 ml of liquid  
paraffin of 60 °C, kept at that temperature during  
5 minutes and then cooled to 5 °C. This temperature  
is maintained during 90 minutes. Subsequently 10 g  
25    of isopropanol are added. The suspended material  
is separated by suction filtration on a membrane  
filter, and washed with isopropanol. Then 3 ml of  
a 10% formaldehyde in isopropanol solution is added  
to the particulate material and reacted during  
30    24 hours at 7 °C. The hardened encapsulated  
material is filtered, washed with PBS and stored  
as a suspension in PBS.

CLAIMS

1. Process for the preparation of immunogens, characterized in that antigens or antigenic determinants or derivatives thereof are bonded to reactive groups of hydrophobic compounds which are complexed with at least one type of glycosides having a hydrophilic and a hydrophobic part, whereafter the resulting immunogenic particles are optionally stabilized and/or protected against digestive degradation.
2. Process according to claim 1, characterized in that two or more different types of antigens and/or antigenic determinants or derivatives thereof are bonded to said reactive group.

3. Process according to claim 1 or 2, characterized in that the immunogenic particles are stabilized by cross-linking of the antigens and/or antigenic determinants or derivatives thereof.
4. Process according to claim 1-3, characterized in that the immunogenic particles are protected against digestive degradation by encapsulation.
5. Vaccine comprising at least one type of immunogen prepared according to one of the processes of the claims 1-4.
6. Immunochemical reagent comprising at least one type of immunogen prepared according to one of the processes of the claims 1-4.



European Patent  
Office

# EUROPEAN SEARCH REPORT

**0142193**  
Application number

EP 84 20 1502

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.)
A	<p>EP-A-0 056 249 (NEW YORK BLOOD CENTER, INC.)</p> <p>* Claims 1,14; page 13, line 4 - page 15, line 4; page 15, lines 9-10,15-35 *</p> <p>---</p>	1-6	<p>A 61 K 39/385 C 07 G 3/ 00 C 07 K 17/ 10 G 01 N 33/548</p>
A	<p>GB-A-2 093 039 (NATIONAL RESEARCH DEV. CORE.)</p> <p>* Claims *</p> <p>---</p>	1-6	
A	<p>NATURE, vol.290, March 5, 1981, pages 51-54, CHESHAM, BUCKS (GB) J. SKELLY et al.: "Hepatitis B polypeptide vaccine preparation in micelle form".</p> <p>* Page 51, right-hand column, lines 3- 21 *</p> <p>---</p>	1-6	<p>TECHNICAL FIELDS SEARCHED (Int. Cl.4)</p> <p>A 61 K C 07 H</p>
A	<p>EP-A-0 058 021 (BEECHAM GROUP PLC)</p> <p>* Claims 1,3,4 and 7; page 1, lines 16-29; page 2, lines 19-24; page 4, lines 26-30 *</p> <p>-----</p>	1-6	
The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>06-02-1985</b>	Examiner <b>RIJCKEBOSCH</b>
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			